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DECLARATION

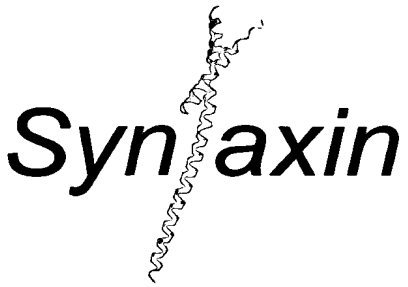
I, the undersigned, Dr Keith Foster, of Syntaxin, Ltd, do solemnly and sincerely declare that I am a technical expert in the field of re-targeted toxin conjugates.

As evidenced by the abridged version of my *curriculum vitae* (Annex 1), I have been actively undertaking research in this technical field for the last 13 years. I am therefore entirely familiar with the field of using re-targeted toxin conjugates in therapeutic methods, in particular, for treating mucus hypersecretion.

I am also familiar with the prosecution history (to date) of US 10/633 698, and in particular with the Examiner' Official Action dated 18 October 2005.

The Examiner has alleged that – of all the possible agents described in the specification of US 10/633 698 - only the specific conjugate WGA-LH_N/A is useful for treating mucus hypersecretion. The Examiner's objection would appear to relate in particular to the suitability of translocating domains other than LH_N.

Translocating domains suitable for use in the agents of the present invention have a common function, in that they deliver the L-chain or L-chain fragment into the cell by a process of endocytosis (see page 4, line 21, of the PCT specification as filed). In this regard, the only functional requirement of a translocation domain of the present invention is that it is capable of releasing the L-chain or L-chain fragment from the endosome – termed "endocytic release".



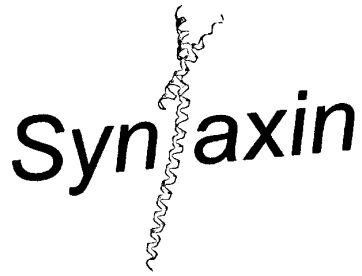
Lord *et al.* (enclosed) provides an overview of internalisation pathways that are routed via the early endosome – in particular, the "H+" internalisation pathway (exemplified by clostridial neurotoxin) and "ER" internalisation pathway (exemplified by PE, shiga toxin and ricin). Whilst these pathways are distinct and have many differences, they share a common route (ie. via the endosome) for translocation of active material into the cytosol. This common route of transportation is well known to those skilled in the art, as are molecules that are internalised by this pathway.

Moreover, as described in more detail below (see my discussion of Shone *et al*, and Blaustein *et al.*) a skilled person would consider it routine to identify molecules that are internalised via the endosomal release pathway.

I would stress in this regard that the present invention does not embrace simple membrane permeable peptides, such as those described in Sandvig *et al.* (enclosed), which transport directly into the cytosol from the extracellular milieu. This "direct delivery" pathway is entirely different from the pathway used by the translocating domains of the present invention, which go through the endosomal release pathway.

As detailed in the present specification, suitable translocation domains may be obtained from a wide range of sources – in particular, microbial sources such as bacterial or viral sources (see page 7, lines 5-6, of the PCT specification as filed). A translocating domain may be an enzyme, such as a bacterial or viral toxin – including the translocating domain of clostridial neurotoxin or diphtheria toxin, or domain II of pseudomonas exotoxin.

Alternatively, as described in the present specification, the translocating activity of the presently claimed agents may be derived from a virally expressed membrane fusion protein. In this regard, a number of examples of suitable proteins are recited on page 7, lines 18-27 of the PCT specification as filed – including influenza virus haemagglutinin, semliki forest virus fusogenic protein, vesicular stomatitis virus glycoprotein G, SER virus F protein and foamy virus envelope glycoprotein.



As described on lines 27-29 on page 7 of the PCT specification as filed, virally encoded "spike proteins" such as the E1 protein of SFV and the G protein of VSV may also provide the requisite translocating activity.

Further examples of molecules that have the desired translocating activity include anthrax PA63 and the *C. botulinum* C_2 domain. As described in Zhang *et al.* (enclosed), anthrax PA63 forms a heptameric channel in the endosome membrane in order to translocate the active components (LF or EF) into the cytosol. Haug *et al.* (enclosed) reports that heptameric pores are also used by the *C. botulinum* C_2 domain (C2II domain) for translocation of the active domain across the endosomal membrane.

Furthermore, a skilled person reading the present specification would be able to identify suitable translocating domains for use in the present invention as a matter of routine. By way of example, suitable methodologies are described in lines 34-37 on page 7; and the second full paragraph of page 9 of the PCT specification as filed (Shone *et al.* and Blaustein *et al.* – copies enclosed).

I make this solemn declaration conscientiously believing the same to be true;

Signed.....

Witnessed ..... C PARSONS .....

Dated ..... 01/02/06 .....

Curriculum Vitae

NAME
Keith Alan Foster

POSITION TITLE
Chief Scientific Officer
Syntaxin Ltd.

Education/Training

INSTITUTION AND LOCATION	DEGREE	YEAR	FIELD OF STUDY
Pembroke College, Cambridge University, UK	M.A.	1977	Natural Sciences, majoring in Biochemistry
St Georges Hospital Medical School, University of London, UK	PhD	1980	Biochemistry

Positions and Employment

1980 - 1982	Post Doctoral Fellow, Department of Biochemistry, Queen's Medical School, University of Nottingham, Nottingham, UK.
1982 - 1993	Principal Scientist,SmithKline Beecham Pharmaceuticals Research Division, Epsom, Surrey, UK.
1993 - 1995	Research Group Leader, The Speywood Laboratory Ltd., London, UK
1995 - 1997	Botulinum Fragments Project Manager & Toxin Biology Unit Head, The Centre for Applied Microbiology & Research (CAMR).
1997 - 2005	General Project Manager, Health Protection Agency (formerly CAMR), Porton Down, Salisbury, Wilts. SP4 0JG, UK
2005 - Present	Chief Scientific Officer, Syntaxin Ltd., Porton Down, Salisbury, Wilts. SP4 0JD, UK

Other Experience and Professional Memberships

1993 - 1995	Honorary Senior Lecturer: Department of Biochemistry, St George's Hospital Medical School, London, UK
1982	Part-time Tutor with the Open University.
1980 - Present	Member of the Biochemical Society
1985 - Present	Member of Society for Medicines Research
1986 - 2003	Member of British Inflammation Research Association
1996 - Present	Member of the Organising Committee for the International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins

Selected peer-reviewed publications (in chronological order).

(Selected from 32 peer-reviewed publications)

1 R.S. Boyd, M.J. Duggan, C.C. Shone & **K.A. Foster**. The effect of botulinum neurotoxins on the release of insulin from the insulinoma cell lines HIT-15 and RINm5F. J. Biol. Chem. 270, 18216-18218 (1995).

2 P. Foran, G.W. Lawrence, C.C. Shone, **K.A. Foster** & J.O. Dolly. BoNT/C1 cleaves both syntaxin and SNAP-25 in intact and permeabilised chromaffin cells: correlation with its blockade of catecholamine release. Biochemistry 35, 2630-2636 (1996).

3 F. Chen, P. Foran, C.C. Shone, **K.A. Foster**, J. Melling & J.O. Dolly. Botulinum neurotoxin B inhibits insulin-stimulated glucose uptake into 3T3-L1 adipocytes and cleaves cellubrevin unlike type A toxin which failed to proteolyze the SNAP-23 present. Biochemistry 36, 5719-5728 (1997).

4 J. Chaddock, J.R. Purkiss, L. Friis, J. Broadbridge, M.J. Duggan, C.C. Shone, C.P. Quinn & **K.A. Foster**. Inhibition of neurotransmitter release by a retargetted endopeptidase derivative of *C. botulinum* neurotoxin A. Infection & Immunity, 68, 2587-2593 (2000).

5 J.A. Chaddock, J.R. Purkiss, M.J. Duggan, C.P. Quinn, C.C. Shone & **K.A. Foster**. A conjugate composed of nerve growth factor coupled to a non-toxic derivative of *Clostridium botulinum* neurotoxin type A can inhibit neurotransmitter release *in vitro*. Growth Factors, 18, 147-155 (2000).

6 J.A. Chaddock, M.H. Herbert, R. Ling, F.C.G. Alexander, S.J. Fooks, D. Revell, C.P. Quinn, C.C. Shone & **K.A. Foster**. Expression and purification of catalytically active, non-toxic derivatives of *Clostridium botulinum* toxin type A. Prot Express Purif., 25, 219-228 (2002).

7 M.J. Duggan, C.P. Quinn, J.A. Chaddock, J.R. Purkiss, F.C.G. Alexander, S. Doward, S.J. Fooks, L. Friis, Y. Hall, E.R. Kirby, N.J. Leeds, H.J. Moulsdale, A. Dickenson, G.M. Green, W. Rahman, R. Suzuki, C.C. Shone and **K.A. Foster**. Inhibition of release of neurotransmitters from rat dorsal root ganglia by a novel conjugate of a *Clostridium botulinum* toxin A endopeptidase fragment and *Erythrina cristagalli* lectin. J. Biol. Chem., 277, 34846-34852 (2002).

8 Chaddock, JA, Duggan, MJ, Quinn, CP, Purkiss, JR, Alexander, FCĜ, Doward, S, Fooks, SJ., Friis, L, Hall, Y, Kirby, ER, Leeds, NJ, Moulsdale, HJ, Dickenson, A, Green, GM., Rahman, W., Suzuki, Rie, Shone, CC and **Foster, KA.** Retargeted clostridial endopeptidases: Inhibition of nociceptive neurotransmitter release in vitro, and antinociceptive activity in in vivo models of pain. Movement Disorders, 19(S8), S42-S47 (2004).

9 **Foster, KA.** The analgesic potential of clostridial neurotoxin derivatives. Expert Opin. Investig. Drugs, 13, 1437-1443 (2004).

10 **Foster, KA.** A new wrinkle on pain relief: re-engineering clostridial neurotoxins for analgesics. Drug Discov. Today, 10, 563-569 (2005).

11 **Foster, KA.** Re-engineering clostridial neurotoxins to create novel, long acting analgesics. In: Toxines et Douleur, Recontres en toxinologie, eds. Bon, C., Goudey-Perriere, F., Goyffon, M. & Sauviat, M-P., p45-54, Lavoisier, France, 2005.

Annex 1

The following conjugates were prepared in accordance with US10/633,698:

1) LH_N/C-EGF (Targeting Domain = epidermal growth factor; Translocation Domain = translocation domain from serotype C from BoNT)

2) LH_N/B-EGF (Targeting Domain = epidermal growth factor; Translocation Domain = translocation domain from serotype B from BoNT)

3) LC/C-RGD-H_N/C (Targeting Domain = RGB; Translocation Domain = translocation domain from serotype C from BoNT)

Each conjugate was then tested for the ability to inhibit mucin release in accordance with Examples 1-3:

Example 1 Effect of LH_N/C-EGF on secreting cell lines
Example 2 Effect of LH_N/B-EGF on secreting cells
Example 3 Effect of LC/C-RGD-H_N/C on secreting cells

The results of Examples 1-3 are illustrated in Figures 1-6:

Figure 1	Effect of LH_N/C-EGF on A549 cells
Figure 2	Effect of LH_N/C-EGF on NCI-H292 cells
Figure 3	Syntaxin cleavage in NCI-H292 cells by LH_N/C-EGF
Figure 4	Mucin levels in A549 cells treated with LH_N/C-EGF
Figure 5	Effect of LH_N/B-EGF on stimulated mucin secretion in A549 cells
Figure 6	Effect of LC/C-RGD-H_N/C on stimulated mucin secretion in A549 cells

Example 1 - Effect of LH_N/C-EGF on secreting cell lines

Methods

Cell cultivation

NCI-H292 (human lung mucoepidermoid carcinoma) cells were seeded into six well plates (Costar) four days prior to use from stocks grown in large 75cm^2 flasks (Nunc). The cells were cultured in RPMI + 10% foetal bovine serum (FBS) + 2mM L-glutamine glutamine in a humidified 5% CO_2 incubator at 37^{0}C. A549 (human lung adenocarcinoma) cells were seeded into six well plates (Costar) four days prior to use from stocks grown in large 75cm^2 flasks (Nunc). The cells were cultured in DMEM + 10% FBS, both obtained from Sigma or another similar provider, + 2mM L-glutamine obtained from Invitrogen or another similar provider. Cells were incubated in a humidified 5% CO_2 incubator at 37^{0}C. In both cases the cells were used at 80% confluence in the wells. Prior to all treatments, the cells were serum-starved for 24 hours.

Inhibition of mucin release following application of LH_N/C-EGF

Both NCI-H292 and A549 cells were treated with various concentrations of fusion molecule for 48 hours in serum-free medium for the respective cell line in a humidified 5% CO_2 incubator at 37^{0}C. The media was removed and fresh serum-free medium containing LH_N/C-EGF was added and the cells incubated plus the stimulant EGF/TNF alpha (20ng/ml and 25ng/ml respectively, both obtained from Sigma or a similar provider) for a further 24 hours and the cells incubated in a humidified 5% CO_2 incubator at 37^{0}C. Following stimulation, the media were collected, stored at -20^{0}C and analysed for mucin content using an enzyme-linked lectin assay (ELLA).

Detection of released mucus in cell culture medium using the enzyme-linked lectin assay (ELLA)

Lectin derived from *Helix pomatia* (edible snail), which binds to specific terminal sugars in mucin glycoproteins, was adhered to a ninety-six well Maxisorp™ plate in phosphate buffered saline (PBS) pH 6.8 for one hour at 37^{0}C. Excess PBS plus lectin were removed from the plate by washing with 0.5 M NaCl, PBS pH7.4. Cell culture medium collected from treated cells was

added and the plate incubated for one hour at 37^{0}C. The plates were then washed in 0.5 M NaCl, PBS pH 7.4 before adding Horseradish Peroxidase conjugated *Helix pomatia* lectin in PBS pH.7.4. Following a further wash step, the assay was developed using OPD (o-phenylenediamine dihydrochloride) peroxidase substrate for ten minutes at 37^{0}C. The reaction was terminated with 2% H_2SO_4 (final concentration) and the absorbance at 492 nm read using a plate reader. Standard curves using human mucin prepared from sputum samples according to literature precedence (Sheehan.,*et al* 1995 *Am. J. Respir. Cell. Mol. Biol.* 13: pp748-756) and medium only controls were included on each Maxisorp™ plate. All chemicals were obtained from Sigma or a similar provider.

Detection of mucin release and cell mucin content using Western blotting technique

Cells treated with LH_N/C-EGF and stimulated were lysed in buffer and run on an agarose gel, transferred to nitrocellulose membranes and probed with Muc5AC monoclonal antibodies (Kirkham., *et al* 2002 *Biochem. J.* 361: pp537). The bands were detected using secondary antibodies conjugated to horseradish peroxidase obtained from Sigma or a similar provider and the resultant band densities were scanned. Media from treated cells was also analysed using this method.

Results

Inhibition of mucus release following application of LH_N/C-EGF

Figure 1 shows a concentration dependent inhibition by LH_N/C-EGF, of mucus release from A549 cells following treatment, with an IC_{50} of 0.4nM. Data shown is from seven independent experiments. Figure 2 shows that the fusion LH_N/C-EGF also inhibited mucin release in a concentration dependent manner from NCI-H292 cells. Data from these two different cell lines indicates that the fusion correctly translocates into the target cells and blocks secretion of mucin from the cells.

Effect of LH_N/C-EGF on mucin content in treated cells.

LH_N/C-EGF blocks release of mucin from stimulated cells (Figures 1 & 2) through cleavage of the SNARE protein syntaxin (Figure 3). Concomitant with a decrease in mucin release with increasing EGF-LHN/C concentration there is an increase in the amount of mucin detected in the treated cells. Figure 4 shows from two independent experiments that the level of mucin remaining in treated cells (intracellular mucin) increases concomitantly with a reduction in the level of mucin released into the medium following a concentration dependent manner.

Example 2 - Effect of LH_N/B-EGF on secreting cells

See Example 1 for methods of cell cultivation and released mucin detection. The cells were treated with LH_N/B-EGF or vehicle or medium alone for 48 hour followed by a 24 hour stimulation of the cells with EGF/TNF alpha (Stimulus) in the presence of the fusion. Vehicle treated cells received fresh vehicle plus medium only. Figure 5 shows a dose-response curve for LH_N/B-EGF in A549 cells. There is a concentration dependent inhibition of mucin release from these cells following treatment.

Example 3 - Effect of LC/C-RGD-H_N/C on secreting cells

See Example 1 for methods of cell cultivation and mucin detection. A549 cells were treated for 48 hours with various concentrations of LC/C-RGD-H_N/C followed by stimulation of cells using EGF and TNF alpha (20ng/ml and 25ng/ml respectively) for 24 hours in the presence of LC/C-RGD-H_N/C. Figure 6 shows a concentration dependent decrease in mucin release. The RGD Targeting Domain enables the LC/C to enter the cells thereby inhibiting mucin release.

Figure 1 - Effect of LH_N/C-EGF on A549 cells

Cells were treated with the fusion or vehicle or medium alone for 48hr followed by a 24hr stimulation of the cells with EGF/TNF alpha (Stimulus) in the presence of the construct. Vehicle treated cells received fresh vehicle plus medium only. Media was collected and assayed for mucin content using the ELLA method with a human mucin standard curve. All mucin levels are shown in ng/ml calculated from the human mucin standard curve within each ELLA

plate. Each concentration was assessed in triplicate and the figure is representative of at least three experiments. N = 7

C = control (cell culture medium only)

VC = vehicle control (50mM Hepes, 200mM NaCl – eluant solution for the LH_N/C-EGF)

**p<0.01, ***p<0.001 *vs* C; ##p<0.01 *vs* 0nM LH_N/C-EGF

Figure 2 - Effect of LH_N/C-EGF on NCI-H292 cells

NCI-H292 cells were treated with the fusion or vehicle or medium alone for 48 hr followed by a 24hr stimulation of the cells with EGF/TNF alpha (Stimulus) in the presence of the fusion. Vehicle treated cells received fresh vehicle plus medium only. Media was collected and assayed for mucin content using the ELLA method with a human mucin standard curve. All mucin levels are shown in ng/ml calculated from the human mucin standard curve within each ELLA plate. Each concentration was assessed in triplicate and the figure is representative of at least three experiments. N = 3

C = control (cell culture medium only)

VC = vehicle control (50mM Hepes, 200mM NaCl – eluant solution for the LH_N/C-EGF)

** p < 0.01 vs C, ## p < 0.01 vs 0nM LH_N/C-EGF

Figure 3 - Syntaxin cleavage in NCI-H292 cells by LH_N/C-EGF

Protein from cells treated for three days with the construct was analysed by Western blot. The Western blot shows the dose-dependent appearance of the cleavage product of syntaxin due to increasing concentrations of LH_N/C-EGF. The blot was probed using a polyclonal antibody raised to the AVKY sequence at the BoNT/C cleavage site in syntaxin. LH_N/C alone or with EGF is not internalised to cause detectable cleavage of syntaxin. EGF alone did not cause cleavage of syntaxin.

Figure 4 - Mucin levels in A549 cells treated with LH_N/C-EGF

Band density on the nitrocellulose blots was detected using a densitometer and has been plotted in Optical density units x area. As the LHN/C-EGF concentration increases the level of mucin detected in the medium decreases

and the intracellular, cell lysate levels increase. Data is representative of two experiments.

C = control (cell culture medium only)

Stimulation = EGF & TNF alpha (20ng/ml & 25ng/ml respectively).

Figure 5 - Effect of LH_N/B-EGF on stimulated mucin secretion in A549 cells

Cells were treated with LH_N/B-EGF or vehicle or medium alone for 48 hour followed by a 24 hour stimulation of the cells with EGF/TNF alpha (Stimulus) in the presence of the construct. Vehicle treated cells received fresh vehicle plus medium only. Medium was collected and assayed for mucin content using the ELLA method with a human mucin standard curve. All mucin levels are shown in ng/ml calculated from the human mucin standard curve within each ELLA plate. Each concentration was assessed in triplicate. LH_N/B-EGF concentration in nM.

C = control (cell culture medium only)

Stim = EGF & TNF alpha (20ng/ml &25ng/ml respectively)

VC = vehicle control (50mM Hepes, 200mM NaCl – eluant solution for the LH_N/B-EGF)

Stimulation = EGF & TNF alpha (20ng/ml &25ng/ml respectively)

Figure 6 - Effect of LC/C-RGD-H_N/C on stimulated mucin secretion in A549 cells

A549 cells treated for 48 hr with 1nM LC/C-RGD-H_N/C then stimulated for 24 hr with EGF/TNF alpha in the continued presence of LC/C-RGD-H_N/C showed a dose-dependent inhibition of mucin release as measured using the ELLA. Each concentration was assessed in triplicate and the figure is representative of two experiments.

C = control (cell culture medium only)

S = EGF & TNF alpha (20ng/ml &25ng/ml respectively)

Stimulation = EGF & TNF alpha (20ng/ml &25ng/ml respectively)

Figure 1 - Effect of LH_N/-EGF on A549 cells

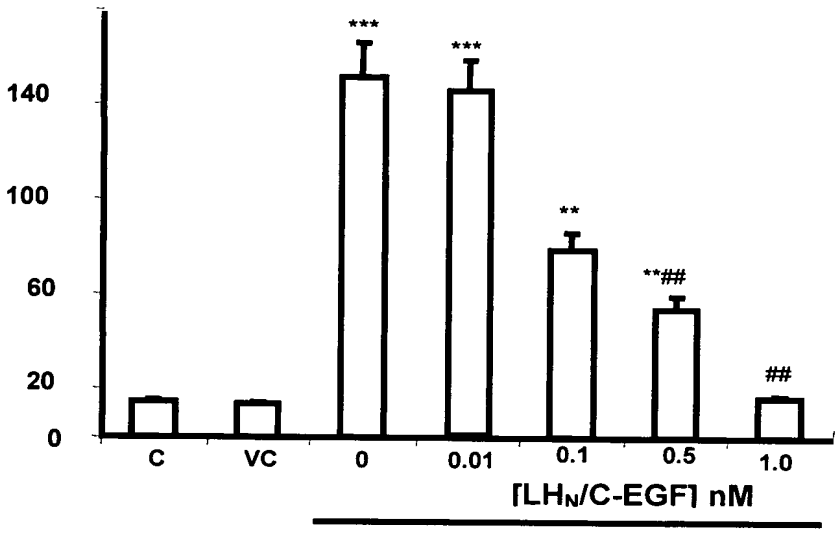


Figure 2 - Effect of LH_N/C-EGF on NCI-H292 cells

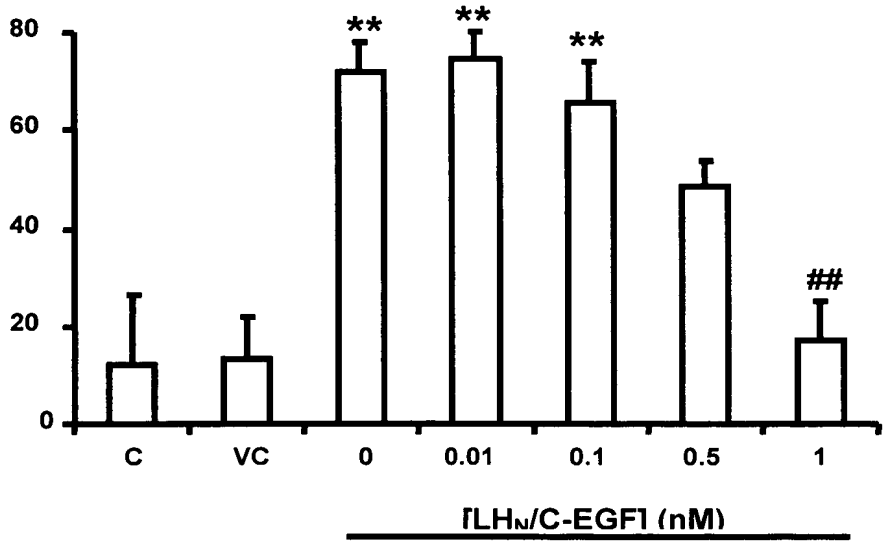


Figure 3 - Syntaxin cleavage in NCI-H292 cells by LH_N/C-EGF

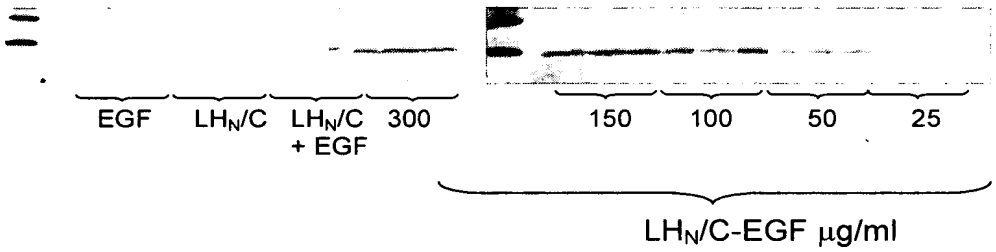


Figure 4 - Mucin levels in A549 cells treated with LH_N/C-EGF

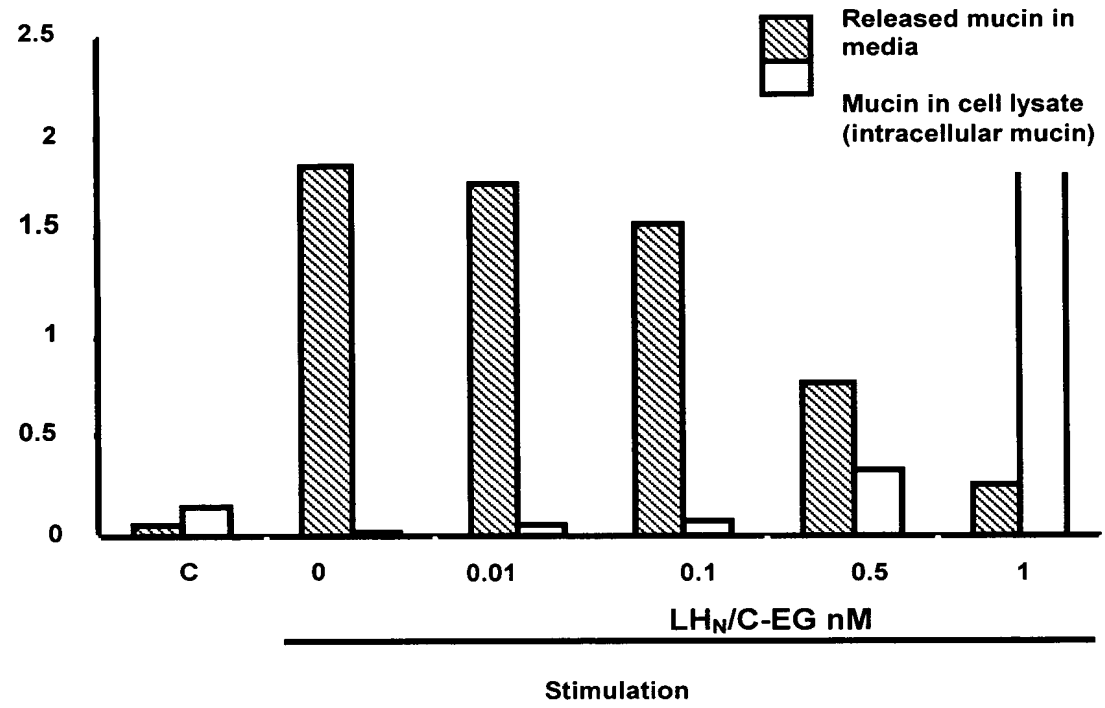


Figure 5 - Effect of LH_N/B-EGF on stimulated mucin secretion in A549 cells

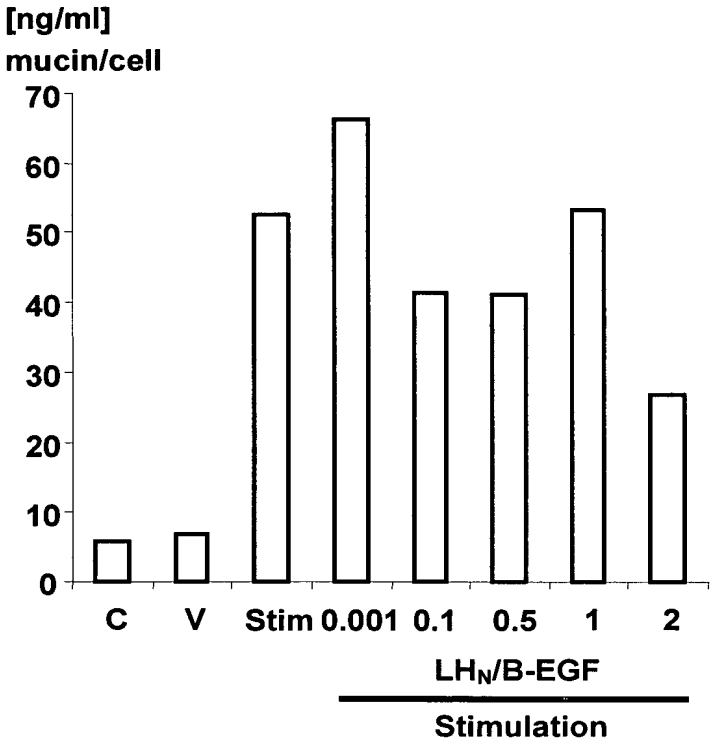


Figure 6 - Effect of LC/C-RGD-H_N/C on stimulated mucin release in A549 cells